**ID1 Promotes Breast Cancer Metastasis by S100A9**

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Abstract

Metastasis is a major factor responsible for mortality in patients with breast cancer. Inhibitor of DNA binding 1 (Id1) has been shown to play an important role in cell differentiation, tumor angiogenesis, cell invasion, and metastasis. Despite the data establishing Id1 as a critical factor for lung metastasis in breast cancer, the pathways and molecular mechanisms of Id1 functions in metastasis remain to be defined. Here, we show that Id1 interacts with TFAP2A to suppress S100A9 expression. We show that expression of Id1 and S100A9 is inversely correlated in both breast cancer cell lines and clinical samples. We also show that the migratory and invasive phenotypes in vitro and metastasis in vivo induced by Id1 expression are rescued by reestablishment of S100A9 expression. S100A9 also suppresses the expression of known metastasis-promoting factor RhoC activated by Id1 expression. Our results suggest that Id1 promotes breast cancer metastasis by the suppression of S100A9 expression.

**Implications:** Novel pathways by Id1 regulation in metastasis. *Mol Cancer Res; 1–10. ©2014 AACR.*

Introduction

Metastasis is a major cause of breast cancer patient death (1–9). Id1 (inhibitor of DNA binding 1) has been found to play critical roles in breast cancer lung metastasis (10–13). Id1 is a member of the Id helix–loop–helix (HLH) transcription factor family (14–24). HLH transcription factors contain a highly conserved HLH domain that mediates homo or hetero-dimerization (14, 20, 24). Most HLH proteins, except for the Id family proteins, also contain a highly basic DNA-binding region adjacent to HLH domain (14, 20, 24). Id transcription factors do not bind DNA but instead regulate gene expression by dimerization with other transcription factors, including both HLH and non-HLH proteins (14, 20, 24). Although most HLH proteins positively regulate gene expression, Id family proteins serve as dominant negative regulators of gene expression (14–24) and play important roles in cell development, including cell differentiation and cell fate determination (19, 22–24). In addition, Id family proteins have also been found to be involved in tumor development (14–18, 20–22). Id1 has been shown to immortalize rodent fibroblasts with Bcl-2 (25). Downregulation of Id2 promotes metastasis in hepatocellular carcinoma (26), and Id1 and Id3 are required for tumor angiogenesis and vascularization in mouse models (27). Id1 overexpression in mammary epithelial cells and breast cancer cells promotes cell invasion and lung metastasis in breast cancer (10–13, 28), whereas downregulation of Id1 expression decreases breast cancer cell invasion and metastasis (29, 30), thus making Id1 also a cancer therapeutic target (18, 29, 31–33). Id1 expression has been shown to be regulated by multiple transcription factors, including sex steroid hormones and the NF-1/Rb/HDAC-1 transcription repressor complex, in breast cancer cells (13, 34, 35). Despite the important functions of Id1 in cancer development, the gene expression and molecular pathways regulated by Id1 in metastasis have not been determined.

We have previously shown that metastasis suppressor KLF17 suppresses Id1 expression in breast cancer (36). Knockdown of KLF17 activates Id1 expression and cell invasion and metastasis (36). However, the molecules and pathways downstream of Id1 that mediate metastasis function are unknown. Although small molecules and peptides have been found to suppress Id1 functions (37–40), both KLF17 and Id1 are transcription factors and considered difficult to target directly. Thus, it is critical to elucidate the genes and pathways that are regulated by Id1 and may mediate its metastasis-promoting functions. In this study,
we show that Id1 promotes metastasis to the lung by suppression of S100A9 expression.

Materials and Methods

Transwell migration and invasion assay

*In vitro* cell migration assays were performed as described previously (36, 41) using Transwell chambers (8 μm pore size; Costar). Cells were allowed to grow to subconfluence (~75%–80%) and were serum starved for 24 hours. After detachment with trypsin, cells were washed with PBS, resuspended in serum-free medium, and 250 μL cell suspensions (2 × 10⁵ cells mL⁻¹) were added to the upper chamber. Complete medium was added to the bottom wells of the chambers. The cells that had not migrated were removed from the upper face of the filters using cotton swabs, and the cells that had migrated to the lower face of the filters were fixed with 5% glutaraldehyde solution and stained with 0.5% solution of Toluidine Blue in 2% sodium carbonate. Images of three random × 10 fields were captured from each membrane and the number of migratory cells was counted. The mean of triplicate assays for each experimental condition was used. Similar inserts coated with Matrigel were used to determine invasive potential in the invasion assay.

Lentivirus transfection and transduction

To generate MCF7 cells stably overexpressing ID1, ID2, and S100A9, respective full-length human cDNAs were cloned into lentiviral vector. Lentivirus was produced by cotransfecting subconfluent human embryonic kidney 293T cells with cDNA expression plasmid and packaging plasmids (pMDLg/pRRE and RSV-Rev) using Lipofectamine 2000. Infectious lentiviruses were collected 48 hours after transfection, centrifuged to remove cell debris, and filtered through 0.45 μm filters using cotton swabs, and the cells that had migrated to the lower face of the filters were fixed with 5% glutaraldehyde solution and stained with 0.5% solution of Toluidine Blue in 2% sodium carbonate. Images of three random × 10 fields were captured from each membrane and the number of migratory cells was counted. The mean of triplicate assays for each experimental condition was used. Similar inserts coated with Matrigel were used to determine invasive potential in the invasion assay.

Immunoblotting

Standard methods were used for Western blotting. Cells were lysed with lysis buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 0.5% Triton X-100, 0.25% sodium deoxycholate, 1 mmol/L EGTA, pH 8.0, 50 mmol/L sodium fluoride, 1 mmol/L sodium vanadate, and 10 mmol/L sodium pyrophosphate with complete protease inhibitor; Roche) and total protein contents were determined by the Bradford method. Of note, 30 μg of proteins were separated using 4% to 12% SDS-PAGE gels and transferred to polyvinylidene difluoride membrane (Millipore). Membranes were incubated overnight at 4°C with specific primary antibodies. Blots were washed and probed with respective secondary peroxidase-conjugated antibodies for 1 hour at room temperature, and the bands visualized by chemoluminescence (Amersham Biosciences). The following antibodies were used: mouse monoclonal S100A9 (R&D Systems), rabbit polyclonal RhoC (Cell Signaling Technology), mouse monoclonal β-actin (Sigma-Aldrich), and secondary mouse peroxidase conjugated (GE healthcare).

Cell proliferation assay

To measure the proliferative activity of cells, each group of cells were seeded in 96-well plates at a density of 4 × 10⁴ cells per well in triplicate. Cell proliferation was quantified at day 0, day 1, day 2, and day 3 with CellTiter 96 AQueous Non-radioactive Cell Proliferation Assay system (Promega) according to manufacturer's protocol. Briefly, cells were incubated with 20 μL of MTS/PMS solution for 4 hours at 37°C in a humidified 5% CO₂ atmosphere. Cell proliferation was then measured at 490 nm using a multiwell plate reader (Wallac Victor, PerkinElmer). Cell growth curves were plotted with optical density value versus time. Three independent experiments were performed.

Immunoprecipitations

For Immunoprecipitation, 200 μg of total cell lysate was incubated with TAF2 antibody and protein G sepharose beads (GE healthcare) overnight at 4°C. The beads with bound protein were pelleted and washed six times with lysis buffer once with ice-cold PBS. The beads were boiled with 1X SDS-PAGE sample buffer. Five percent input and fifty percent immunoprecipitated proteins were separated using 4% to 12% SDS-PAGE gels and Western blotting was
performed as described above. The following antibodies were used: Rabbit polyclonal ID1 (Santa Cruz Biotechnology) and ChIP grade rabbit polyclonal TFAP2A (Abcam) and secondary rabbit peroxidase conjugated (GE healthcare).

RNA isolation, reverse transcription, and RT-PCR analysis
Total RNA was extracted from cell lines, frozen primary and metastasis tissues using TRIzol total RNA isolation reagent (Invitrogen), according to the manufacturer’s specifications and treated with Turbo DNase (Ambion). cDNA was synthesized from total RNA using random hexamers with TaqMan High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene primers were designed using Primer Express v3.0 Software and RT-PCR was performed using SYBR Select Master Mix (Applied Biosystem). All reactions were carried out on the 7500 Fast Real-Time PCR system (Applied Biosystem). The average of three independent analyses for each gene and sample was calculated using the ΔΔ threshold cycle (Ct) method and was normalized to the endogenous reference control gene GAPDH.

Chromatin immunoprecipitation
MCF7 cells or MCF7 cells overexpressing ID1 were fixed in 1% formaldehyde at 37°C for 10 minutes. Cells were washed twice with ice-cold PBS containing protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, and 1 μg/mL pepstatin A), scraped, and centrifuged at 4°C. Cell pellets were resuspended in lysis buffer and sonicated to shear DNA. After sonication, the lysate was centrifuged and the supernatant was diluted 10-fold with chromatin immunoprecipitation (ChIP) dilution buffer (0.01% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, and protease inhibitors). Chromatin–protein complexes were immunoprecipitated using anti-TFAP2A or normal rabbit IgG for overnight at 4°C with rotation. The immunocomplex was precipitated with protein A/G-agarose and washed sequentially with low salt buffer, high salt buffer, and lithium chloride wash buffer and eluted with elution buffer (1% SDS, 0.1 mol/L NaHCO3, and 200 mmol/L NaCl). Reverse of cross-linking was done by heating at 65°C overnight in the presence of NaCl. DNA was purified using PureLink PCR Purification Kit (Invitrogen). The amount of immunoprecipitated DNA was analyzed in triplicates using TFAP2A-binding primer sequence in human S100A9 promoter by RT-PCR on ABI 7500 detection system (Applied Biosystems). Data were analyzed using the percent input method.

Microarray data analysis
Total RNA was extracted using the TRI Reagent (Ambion) method and treated with DNaseI (Fermentas). For microarray analysis, RNA quality was determined using the Bioanalyzer (Agilent) and only samples with RIN numbers >7.5 were used for further studies. Equal amounts of total RNA for each sample (400 ng) were amplified as recommended by Illumina and hybridized to the Human- HT12 v4 human whole-genome bead arrays. Illumina GenomeStudio software was used to export expression levels and detection P values for each probe of each sample. Signal intensity data were quantile normalized and only genes that showed insignificant detection P value (P > 0.05) in all samples were removed from further analysis. The data were submitted to Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) and are available by accession number GSE53847. Unpaired two-tailed t test was used to compare groups and P value was corrected for multiple testing according to Storey and colleagues (42). Only genes that were significant in ID1 versus control comparison (at FDR < 15%), but were insignificant in ID2 versus control comparison (nominal P > 0.05) were considered.

Results
Id1 and Id2 have different functions in metastasis
Our previous study has shown that Id1 is directly regulated by metastasis suppressor KLF17 and that knockdown of KLF17 activates Id1 expression and promotes lung metastasis in breast cancer (36). We have also shown that over-expression of Id1 recapitulates the metastasis phenotype induced by KLF17 knockdown (36). Knockdown of Id1 in human breast cancer cell line MDA-MB-436 did not affect cell growth in vitro (Fig. 1A), but suppressed cell migration and invasion (Fig. 1B and C) in vitro and metastasis in vivo (Fig. 1D). In the gene expression profiling analysis of KLF17, we found that the expression of two Id family members, Id1 and Id2, is regulated by KLF17 (36). To test whether Id2 has similar functions as Id1 in metastasis, we generated luciferase-tagged human breast cancer MCF7 cells stably expressing Id2 (Supplementary Fig. S1) and transplanted these cells into mice mammary fat pads. The primary tumor growth of cells expressing Id1 or Id2 is similar (Fig. 1E). Nine out of 10 mice transplanted with MCF7 cells stably expressing Id2 (Supplementary Fig. S1) did not develop any metastasis (Fig. 1F), whereas mice transplanted with Id1-overexpressing cells (Supplementary Figs. S1 and S2), all (10 out of 10) developed lung metastasis (Fig. 1F). These results demonstrated that Id1 and Id2 have different functions in metastasis even though they share sequence similarities in HLH domain and belong to the same family. Thus, we hypothesized that genes differentially regulated by Id1 but not by Id2 are potential candidates that function in breast cancer metastasis.

S100A9 expression is regulated by Id1
To identify genes that are regulated by Id1 but not Id2, we performed gene expression profiling analysis in MCF7 cell overexpressing Id1 or Id2 and identified S100A9 as the top gene in which expression was suppressed by Id1 but not Id2 (Supplementary Fig. S3). S100A9 is a calcium-binding protein that plays critical roles in inflammation and cancer development (43, 44). The functions of S100A9 in cancer development are cancer type dependent (45–47). In addition, it has been shown that S100A9 has both tumor-
**Figure 1.** Id1 but not Id2 promotes metastasis. A, cell growth of MDA-MB-436 cells expressing Id1 shRNAs. Downregulation of Id1 expression in MDA-MB-436 cells did not affect cell growth. B–D, downregulation of Id1 expression decreased cell migration, invasion, and metastasis. MDA-MB-436 cells stably expressing a control shRNA or Id1 shRNA were subjected to migration assay (B) and invasion assay (C). Knockdown of Id1 expression in MDA-MB-436 cells suppressed cell migration and invasion. D, transplantation of luciferase-tagged MDA-MB-436 cells stably expressing a control shRNA via tail vein injection led to metastasis (8 out of 10 mice developed lung metastasis), whereas transplantation of MDA-MB-436 cells stably expressing Id1 shRNAs did not (only 1 out of 5 mice in each of the Id1 shRNA group developed metastasis; Fisher exact test P = 0.023). Knockdown of Id1 expression in MDA-MB-436 cells suppressed metastasis. E, tumor growth in the primary site after the transplantation of human breast cancer MCF7 cells stably expressing Id1 or Id2. There is no significant difference between two groups. F, transplantation of MCF7 cells stably expressing Id1 in mammary fat pads leads to metastasis (10 out of 10 mice developed lung metastasis), whereas transplantation of MCF7 cells stably expressing Id2 does not (only 1 out of 10 mice developed lung metastasis; Fisher exact test P < 0.001).
promoting and tumor-suppressive functions in breast cancer (48, 49). To confirm the regulation of S100A9 expression by Id1, we determined the expression level of S100A9 by qRT-PCR and immunoblot analysis in MCF7 cells stably expressing Id1 or Id2. S100A9 expression was suppressed by Id1 at both the transcriptional level and protein level, but not by Id2 (Fig. 2A and B). Transient transfection of Id1 or Id2 into MCF7 cells resulted in S100A9 suppression by Id1 but not Id2 (Supplementary Fig. S4A and S4B). Because Id family proteins, including Id1, have been shown to serve as negative regulators of gene expression by binding to other transcription factors (14–24), we then scanned the promoter sequence of S100A9 using JASPAR database for potential binding sites for transcription factors that are known to form heterodimers with Id family members bind. We found four potential TFAP2A-binding sites (site 1: −351 to −343; site 2: −682 to −674; site 3: −1024 to −863, and site 4: −1248 to −1240) in the S100A9 promoter. Coimmunoprecipitation analysis in human breast cancer cell MDA-MB-436 where Id1 is endogenously expressed showed that Id1 interacted with TFAP2A in vivo (Fig. 2C). The interaction of Id1 and TFAP2A was further validated in MCF7 cell overexpressing Id1 (Fig. 2D). To determine whether TFAP2A directly binds to the S100A9 promoters in vivo, we carried out ChIP analysis using control IgG or TFAP2A antibody. Three of the four predicted S100A9 promoter regions with TFAP2A consensus sequences were significantly enriched after immunoprecipitation by an anti-TFAP2A antibody. The enrichment was significantly reduced in site 1, 2, and 3 in MCF7 cells stably expressing Id1.

To further validate the regulation of S100A9 by Id1, we examined the expression of S100A9 and Id1 in breast cancer cell lines and primary human breast cancer samples.
Spearman correlation analysis indicated that the expression of Id1 and S100A9 were inversely correlated in the human breast cancer cell lines (Fig. 3A). We then examined the expression of these two genes in primary human breast cancer samples with known lymph node metastasis status. Spearman correlation analysis indicated that the expression of Id1 and S100A9 was inversely correlated in clinical breast cancer samples (Fig. 3B). Wilcoxon rank-sum test indicated that the mean Id1 expression was significantly higher in patients with lymph node metastasis than the mean expression in patients without metastases (Fig. 3B). In contrast, mean S100A9 expression in patients with lymph node metastasis was significantly lower than the mean expression in patients without metastases (Fig. 3B). These results indicated that the expression of Id1 and S100A9 was inversely correlated in both breast cancer cell lines and clinical samples.

### Id1 promotes metastasis by the regulation of S100A9

To determine whether Id1 promotes metastasis by suppressing S100A9 expression, we overexpressed an S100A9 cDNA in MCF7 cells stably expressing Id1. The expression of S100A9 was confirmed by qRT-PCR (Fig. 4A). S100A9 abrogated the migratory and invasive phenotypes induced by Id1 in the migration and invasion assays *in vitro* (Fig. 4B and C). Transient transfection of Id1 and S100A9 in MCF7 cells led to similar results (Supplementary Fig. S5A–S5C). This was not due to changes in cell growth, as S100A9 expression did not affect cell growth *in vitro* (Supplementary Fig. S6). MCF7 cells stably expressing both S100A9 and Id1 were also transplanted into mammary fat pads in mice. The primary tumor growth of S100A9 and Id1 was similar, only slightly lower at the fourth week when compared with the growth of cells expressing Id1 alone (Fig. 4D). The lung metastasis signals were significantly suppressed in mice with the Id1 and S100A9 double-expressing cells as compared with mice with only expressing Id1 (Fig. 4E). Taken together, these results indicated that suppression of S100A9 by Id1 is responsible, at least in part, for the observed role of Id1 in promoting metastasis.

### S100A9 mediates the regulation of RhoC by Id1

To identify the molecules and pathways downstream of S100A9 in metastasis, we searched the gene expression profiles of MCF7 cells stably expressing Id1 or Id2 and found that known metastasis-promoting gene RhoC was upregulated only in the Id1-expressing cells. We further analyzed RhoC expression in MCF7 cells stably expressing S100A9 and Id1 or Id1 alone by qRT-PCR. Expression of RhoC was increased in the cell expressing only Id1 but was significantly suppressed in cells stably expressing both Id1 and S100A9 (Fig. 5A). These results were further confirmed by the immunoblotting (Fig. 5B). Knockdown of Id1 in MDA-MB-453 cells that express high level of Id1 but low level of S100A9 resulted in the upregulation of S100A9 expression and the downregulation of RhoC expression (Fig. 5C). To determine the effects of RhoC downregulation in migration and invasion, we knocked down RhoC expression using two different shRNAs in MCF7 cells stably expressing Id1. The downregulation of RhoC expression by shRNAs was confirmed by immunoblot analysis (Fig. 5D). Downregulation of RhoC abrogated the migratory and invasive phenotypes induced by Id1 expression in MCF7 cells (Fig. 5E and F). Conversely, overexpression of RhoC in MCF7 cells expressing Id1 and S100A9 partially rescued the migratory and invasive phenotypes that were...
suppressed by S100A9 (Fig. 5G and H). These results indicated that RhoC expression is regulated by S100A9 and RhoC suppression reduced the migratory and invasive phenotypes induced by Id1 expression.

Discussion

We have previously reported that knockdown of metastasis suppressor KLF17 activates Id1 expression and promotes lung metastasis in breast cancer. However, the molecular pathways downstream of Id1 were not known. In this study, we show that Id1 promotes metastasis by the suppression of S100A9 expression in breast cancer cells. We also found that RhoC mediates Id1 functions and is downstream of S100A9. On the basis of these results, we propose a molecular pathway model for the function of KLF17: Knockdown of KLF17 activates Id1 expression, which interacts with TFAP2A to suppress S100A9 expression. The
Figure 5. S100A9 suppresses RhoC expression. RhoC expression in MCF7 cells stably expressing a vector control, or Id1, or Id1 and S100A9 was determined by quantitative RT-PCR (A) and immunoblot analysis (B). Id1 expression in MCF7 cells increased RhoC expression (A and B). Expression of S100A9 cDNA reversed the increase of RhoC expression induced by Id1 expression (A and B). C, immunoblot analyses of Id1, S100A9, and RhoC in MDA-MB-436 cells expressing a control shRNA or Id1 shRNA. Knockdown of Id1 expression in MDA-MB-436 cells increased S100A9 expression and decreased RhoC expression. D, RhoC expression in MCF7 cells expressing a vector control, or Id1, or Id1 and RhoC shRNA 1, or Id1 and RhoC shRNA 2. E–H, RhoC mediated the Id1 functions in migration and invasion. MCF7 cells stably expressing a vector control, or Id1, or Id1 and RhoC shRNA 1, or Id1 and RhoC shRNA 2 were subjected to migration (E) and invasion (F) assays. Id1 expression in MCF7 cells increased cell migration and invasion (E and F). Knockdown of RhoC expression reversed the migratory and invasive phenotypes induced by Id1 (E and F). MCF7 cells stably expressing a vector control, or Id1, or Id1 and S100A9, or Id1 plus S100A9 and RhoC were subjected to migration (G) and invasion (H) assays. S100A9 expression decreased the cell migratory and invasive phenotypes induced by Id1 (G and H). Expression of RhoC rescued the suppressive phenotype of S100A9 in migration and invasion (G and H).
Id1 Promotes Metastasis by Suppressing S100A9

suppression of S100A9 leads to the activation of RhoC, cell migration, invasion, and metastasis phenotypes. S100A9 expression partially rescued the metastasis phenotype induced by Id1, thus it is likely that there are other molecules regulated by Id1 that also participate in the Id1 metastasis function. The identification and characterization of these molecules are currently underway.

Id family members share similar sequences and form heterodimers with other transcription factors to regulate gene expression. Although it is well established that Id1 is a major promoting factor for lung metastasis in breast cancer, downstream target genes and molecular pathways have been elusive. This is at least partly due to the factor that Id family members interact with other transcription factor to regulate gene expression instead of directly binding to the promoters of the genes they regulate. They regulate a large number of genes and overlap in the target gene regulation. Our results indicated that different Id family members have different functions and the downstream target genes that mediate these functions are also differentially regulated. Our findings that Id1 but not Id2 promotes lung metastasis in breast cancer also led us to identify S100A9, an important player in the regulation of metastasis functions of Id1. Id family members have diverse functions in development and tumorigenesis. The genes and molecule pathways regulated by Id family members in these processes are largely unknown. Our approach can be used to identify these additional genes and pathways that mediate the functions of Id family members.

S100A9 functions in a variety of physiologic process, including inflammation and myeloid cell maturation. It binds to cell surface receptors, including TLR4/RAGE, and initiates signaling pathways that affects multiple cellular functions, including the activation of reactive oxygen species pathway. It has also been shown that S100A9 can promote or suppress breast cancer development (48, 49). The dichotomy of S100A9 functions in breast cancer possibly depends on cellular context, local concentration, its expression level, and posttranslational modification. It has been shown to promote metastasis in melanoma (47), but suppresses metastasis in human cervical cancer (46). Our results indicated that S100A9 is regulated by Id1 and serves as a negative regulator of RhoC, a critical factor in cell motility and metastasis. The molecular mechanisms of RhoC regulation by S100A9 in breast cancer remain to be determined. The other aspect that is important in the understanding of S100A9 regulation of metastasis is whether the expression of S100A9 in cancer cells also affects tumor microenvironment in breast cancer. The characterization of S100A9 in both cancer cells and tumor microenvironment will further improve our knowledge of S100A9 in cancer development.

S100 family members have diverse functions in cancer development. Some of the family members have overlapping functions and mechanisms in gene regulation. We found that S100A7 and S100P are also differentially regulated by Id1 but not Id2. However, these two genes do not mediate the metastasis functions of Id1 (data not shown). Understanding the differential functions of S100 family members in different steps of cancer development is critical to our understanding of the roles of this protein family. Both KLF17 and Id1 are transcription factors that are difficult to target by small molecules. The discovery of the downstream targets of Id1 that mediate its functions may provide potential therapeutic targets for the suppression of tumor metastasis in this pathway.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: K. Gumireddy, J. Yan, Q. Huang
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